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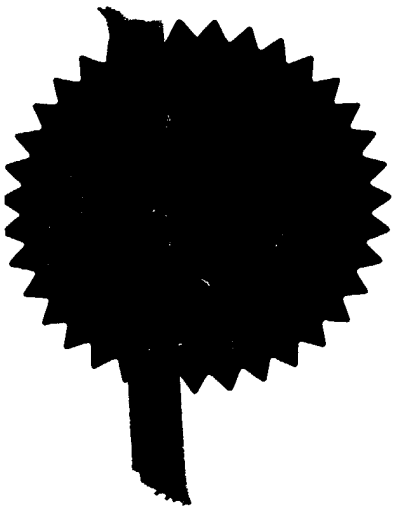

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1. Your reference — 2475/02623

2. Patent application number *(The Patent Office will fill in this part)* — **9915226.6**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Knoll AG
Post Box 210805
Ludwigshafen

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — Germany D-67008

4. Title of the invention

Screening Method

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Dr T K Miller
Knoll Pharmaceuticals
Patents Department
Research R4
Pennyfoot Street
Nottingham NG1 1GF

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Signature J. Miller Date 29/6/99

12. Name and daytime telephone number of person to contact in the United Kingdom — Dr T K Miller 0115 912 4286

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Screening Method

The present invention relates to a novel regulatory site that leads to an increase in the activity of the mitochondrial proton leak, and to the use of this site in a novel screening method for compounds which are useful in the treatment of body weight disorders and related co-morbid conditions, including, but not limited to, obesity, diabetes and dyslipidaemias.

Oxidative phosphorylation is the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i) by mitochondria driven by an electrochemical proton gradient that is established during the flow of electrons through the respiratory chain. During electron transfer, protons are pumped from the mitochondrial matrix into the intermembrane space. Protons diffuse back into the matrix through the ATP synthase producing ATP. However, not all of the proton back-flow occurs through ATP synthase. When oligomycin (an inhibitor of proton flow through the membrane domain of ATP synthase) is added to isolated mitochondria, the mitochondria still continue to consume oxygen at a low rate. This process is known as the "proton leak " (Brand et al, Biochimica et Biophysica Acta 1187 (1994) 132-139). This proton leak across the mitochondrial inner membrane is estimated to account for as much as 35-50% of skeletalmuscle respiration (Rolfe D.F.S. and Brand M.D. (1996) *Am. J. Physiol.* 271S C1380-C1389). In the absence of oxidative phosphorylation all the protons pumped by the respiratory chain out of the mitochondria return into the mitochondria by this proton leak.

The mitochondrial proton leak constitutes a significant proportion of the basal metabolic rate of an organism. Hitherto, there have been no known acutely acting regulators of this biological process. However, an increase of 15% in skeletal muscle mitochondria respiration rate (state 4) caused by supraphysiological concentrations (4.16 mM) of cytidine monophosphate (CMP) has recently been reported (Jekabsons M. and Horwitz B.A. (1998) *FASEB J.* 12, n° 5, part II, 4714). The authors suggested a possible CMP regulation of proton leak in skeletal muscle mitochondria. However, our results did not confirm this effect at concentrations of up to 1mM. In our systematic examination of the effects of fifteen nucleotides on

skeletal muscle mitochondria it has surprisingly been found that only one nucleotide had an effect at physiologically relevant concentrations.

The present invention provides a strategy for screening for compounds that activate the mitochondrial proton leak by interacting at this novel regulatory site. Compounds shown to activate the proton leak can now be tested in the presence or absence of AMP. A lack of an additive effect indicates that the compound was interacting at this novel site. Compounds which activate this novel AMP regulated proton leak will increase the basal metabolic rate and hence may be useful in treating obesity and related conditions.

The present invention provides a screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system ;

b) measuring the oxygen consumption; and

c) identifying compounds which increase oxygen consumption.

Preferably the method further comprises the steps of a) contacting the compounds identified with mitochondria in the presence of AMP and measuring the oxygen consumption; and b) comparing the oxygen consumption in the presence of AMP and the absence of AMP and thus identifying compounds where there is not an additive effect on oxygen consumption as compounds which activate the AMP-sensitive regulatory site.

The present invention also provides a screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;

b) measuring the membrane potential; and

c) identifying compounds which decrease the membrane potential.

Preferably the method which further comprises the steps of a) contacting the compounds identified with isolated mitochondria in the presence of AMP and

measuring membrane potential, and b) comparing the membrane potential in the presence of AMP and the absence of AMP and thus identifying compounds where there is not an additive effect on membrane potential as compounds which activate the AMP-sensitive regulatory site.

The present invention also provides a screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;

b) measuring the oxygen consumption and measuring the membrane potential; and

c) identifying compounds which increase oxygen consumption and which decrease the membrane potential.

Preferably the method further comprises the steps of a) contacting the compounds identified with mitochondria in the presence of AMP and measuring the oxygen consumption and measuring the membrane potential; and b) comparing the oxygen consumption and the membrane potential in the presence of AMP and the absence of AMP and thus identifying compounds where there is not an additive effect on oxygen consumption and the membrane potential as compounds which activate the AMP-sensitive regulatory site.

Suitably, the mitochondria are isolated mitochondria or are present in intact cells. Preferably, the mitochondria are isolated skeletal muscle mitochondria. More preferably the mitochondria are isolated rat skeletal muscle mitochondria. Preferably the mitochondria are present in intact eukaryotic cells or are present in tissue slices of mammalian origin or cell lines of mammmalian origin.

Suitably any substrate for respiration may be used. Preferably the substrate for respiration is a succinate salt, a glutamate salt or a malate salt, for example as a potassium salt or a sodium salt. More preferably, the substrate is a succinate salt, for example potassium succinate or sodium succinate. Preferably the screening method is carried out in the presence of an inhibitior of the utilisation of other endogenous substrates (a complex 1 inhibitor). When a succinate salt is employed

then the screening method is preferably carried out in the presence of rotenone to inhibit the utilisation of other endogenous substrates.

Preferably, the screening method is carried out in varying concentrations of an electron transport inhibitor. More preferably, the electron transport inhibitor is selected from a malonate salt, myxothiazol or a cyanide salt. Most preferably, the electron transport inhibitor is a malonate salt, for example the sodium or the potassium salt.

When membrane potential is being measured then the screening method is preferably carried out in the presence of a proton/potassium exchanger, for example nigericin, to minimise the pH gradient.

Membrane potential is preferably measured using a) ion selective electrodes for methyltriphenylphosphonium cation (TPMP) (or tetraphenylphosphonium cation (TPP)) wherein TPMP (or TPP) has been added to the test system or b) by using fluorescent membrane potential dyes wherein changes in membrane potential are measured by a fluorimeter which records the changes in the fluorescent response due to partitioning. A suitable dye is (dimethyl(aminostyryl)-1-methyl pyridinium (DSMP)) which may be used to measure membrane potential in both intact cells and isolated mitochondria.

Preferably the oxygen consumption and/or the membrane potential measurements are carried out in the presence of an inhibitor of ATP synthesis for example oligomycin.

Preferably the oxygen consumption is measured using an oxygen electrode.

The term buffer system is used herein to mean a system capable of supporting mitochondria and comprises a buffering agent, for example HEPES, and an osmotic protector, for example KCl. The buffering system optionally further comprises a chelating agent, for example EGTA, and/or an inorganic phosphate, for example potassium dihydrogen phosphate, and/or a free fatty acid scavenger, for example defatted BSA.

The term "associated co-morbid conditions" as used in this document means medical conditions known to those skilled in the art to be associated with body weight disorders. The term includes but is not limited to the following: diabetes including non-insulin dependent diabetes mellitus, impaired glucose tolerance, lipid syndromes, hyperglycaemia and hyperlipidaemia, high uric acid levels and lipid levels, in mammals particularly humans.

In addition the present invention may be useful in identifying compounds for the treatment or prevention of metabolic diseases and conditions arising therefrom, for example non exercise activity thermogenesis and increased metabolic rate, weight gain associated with drug treatment, osteoarthritis and gout, cancers associated with weight gain, menstrual dysfunction or gallstones.

The present invention may be useful in identifying compounds for preventing cardiovascular disease, in aiding weight loss after pregnancy and in aiding weight loss after smoking cessation.

In another aspect the present invention provides a protein which is involved in the AMP activated proton leak. In yet another aspect the present invention provides methods for identifying this protein or proteins. The term "involved in" covers proteins on which AMP has a direct action and which directly affects the proton leak and proteins which interact with AMP and then affect the proton leak by indirect means.

A first method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

a) contacting AMP or an analogue thereof with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;

b) measuring the oxygen consumption and/or the membrane potential;

c) contacting AMP or an analogue thereof with mitochondria in the presence of a substrate for respiration in the presence of a buffer system in the presence of a known specific inhibitor of a mitochondrial protein;

d) measuring the oxygen consumption and/or the membrane potential;

e) identifying a protein whose inhibitor reduces the increase in oxygen consumption and/or the decrease in membrane potential caused by AMP as a protein which is involved in the AMP activated proton leak.

The term "an analogue thereof" means a compound which has a similar effect on the proton leak as AMP or has higher affinity.

It will be understood by those skilled in the art that the necessary control experiments are required to validate this method. For example it would be desirable to incubate the mitochondria with the specific protein inhibitor to ensure that there was no direct effect of the protein inhibitor on the proton leak. It would also be desirable to incubate the mitochondria in the buffer system as a control.

A second method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

a) contacting AMP or an analogue thereof with mitochondria of modified protein composition in the presence of a substrate for respiration in the presence of a buffer system;

b) comparing the effect of AMP or a high affinity analogue on oxygen consumption and/or the membrane potential on mitochondria with modified protein composition with control mitochondria;

c) identifying modifications in protein composition which affect the increase in oxygen consumption and/or the decrease in membrane potential caused by AMP and hence identifying the corresponding protein/s which are involved in the AMP activated proton leak.

The mitochondria of modified protein composition may be obtained by methods known to those skilled in the art, for example, the mitochondria may be collected from animals which have been subjected to a stress eg overfeeding , underfeeding, heat, cold , restricted movement etc. or whose environment has been otherwise manipulated. Alternatively dosing of animals with pharmacological agents may be used to alter mitochondrial protein composition. Alternatively the mitochondria may be collected from genetically modified strains of animals.eg ob/ob mice, Zucker rats and strains in which certain genes and proteins have been inactivated by generic means including deletions of mitochondrial genes.

A third method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

a) extracting the proteins from mitochondria and separating and purifying these proteins by methods known to those skilled in the art;

b) incubating the separated proteins with a fluorescent, radiolabelled or other labelled AMP or analogue in a suitable binding assay using methods known to those skilled in the art;

c) identifying those pure proteins which bind labelled AMP or analogue as being proteins involved in the AMP activated proton leak and

d) partially sequence the purified protein and using techniques known to those skilled in the art construct probes to allow the gene to be identified.

A fourth method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

a) extracting the proteins from mitochondria and incubating them with a photoaffinity labelled version of AMP or analogue using methods known to those skilled in the art to label proteins;

b) thereafter extracting, isolating and purifying those labelled proteins by methods known to those skilled in the art

c) identifying the structure of these proteins; and

d) identifying these proteins as proteins involved in the AMP activated proton leak; and

e) partially sequence the purified protein and using techniques known to those skilled in the art construct probes to allow the gene to be identified.

A preferred method of purifying a protein which is involved in the AMP activated proton leak comprises passing extracted proteins, obtained as described previously, through a column containing immobilised AMP or an analogue thereof.

In a further aspect the present invention provides a screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

a) contacting a test compound with a protein which is involved in the AMP activated proton leak in a protein binding assay and identifying compounds with a high binding affinity as compounds which activate an AMP-sensitive regulatory site on mitochondria.

It will be understood by those skilled in the art that the protein used may be either purified protein or recombinant protein obtained by methods known to those skilled in the art and as described below.

Once the protein has been identified improved assay methods can be developed. For example, the three dimensional structure of active sites on the protein may be identified by crystallography which will increase knowledge of the structure and function. Computer modelling may be used to identify compounds which are likely to interact at these active sites and hence increase the chances of identifying compounds which have useful therapeutic properties. A specific assay could be developed to identify compounds which interact at these active sites.

In addition the target protein may be reconstituted in artificial membrane structures (eg liposomes) to further characterize function and its regulation.

In another aspect the present invention provides a gene which expresses a protein which is involved in the AMP activated proton leak. In yet another aspect the present invention provides a method for identifying this gene.

A method for identifying a gene which expresses a protein which is involved in the AMP activated proton leak comprising the steps of :

a) searching genomic databases and identifying genes which have an AMP binding domain;
b) expressing these genes in cells;
c) isolating the mitochondria from said cells; and
d) determining which mitochondria have an altered response to AMP (when compared to control) and hence determine which of these genes are responsible for the AMP activated proton leak.

Once the gene has been identified improved assay methods may be developed as follows. Firstly an assay method may be utilised in which, once a promoter region of the gene has been identified by methods known to those skilled in the art, the gene may be upregulated to produce additional protein in a target cell thus increasing the sensitivity of the assay. In other words the protein may be overexpressed in a suitable expression system as known to those skilled in the art.

Secondly the AMP regulated proton leak may be generated in cells which do not normally have it and such cells may be used in the screening assay.

In an additional aspect the present invention provides a regulatory site for a mitochondrial proton leak wherein the site is activated by adenosine monophosphate (AMP).

In another aspect the present invention provides the gene identified by the above methods.

In a further aspect the present invention provides the protein which acts as a regulatory site for a mitochondrial proton leak wherein the site is activated by AMP.

In a yet further aspect the present invention provides the protein identified by the methods identified above.

EXPERIMENTAL PROCEDURES

The effect of adenosine, guanosine, cytidine, thymidine and uridine mono- di- and triphosphates at a concentration of 1 mM on the state 4 respiration rate (defined below) and proton leak of rat skeletal muscle mitochondria was studied.

Isolation of skeletal muscle mitochondria.

Female Wistar rats (4-8 wk old) were killed by stunning followed by cervical dislocation and the skeletal muscle was immediately dissected from the hindlimbs, weighed and placed in pre-weighed beaker containing C-P 1 medium (0.1 M KCl, 0.05 M Tris-HCl, 2 mM EGTA, pH 7.4). Mitochondria were isolated according to the methods of Chappell J.B. and Perry S.V. (1954) Nature (London) 173, 1094-1095 and Bhattacharya *et al. (1991)* Anal. Biochem. 192, 344-349. Briefly, the tissue was placed on a pre-cooled porcelain tile and shredded with a sharp blade. The tissue was minced and washed further by chopping with a sharp scissors and rinsing with C-P 1 medium 4-5 times and then drained of medium and left stirring in a beaker on ice containing C-P 2 medium (0.1 M KCl, 0.05 M Tris-HCl, 2 mM EGTA, 1 mM ATP, 5 mM $MgCl_2$, 0.5% bovine serum albumin (BSA), and 18.7 U protease (nagarse)/g tissue, pH 7.4) for 4 min. The tissue was homogenised in the same medium using a

Polytron tissue homogeniser. The homogenised tissue was left stirring in the same medium on ice for a further 6 min and then was centrifuged at 490g for 10 min. The supernatant was filtered through muslin and again centrifuged at 10368g for 10 min. The mitochondrial pellets were resuspended in C-P 1, combined and centrifuged again at 10368g for 10 min. A last centrifugation was performed at 3841g and finally the pellet was resuspended in approx. 500 µl of C-P 1. Protein concentration was determined by the Biuret method.

Measurement of Oxygen Consumption.

The respiration rate was measured in the absence of adenosine diphosphate (ADP) (state 4) and presence of oligomycin (to inhibit any ATP synthesis) as a crude indicator of mitochondrial proton conductance. Oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech, Great Britain) maintained at 37°C. Prior to any experimental runs, the linearity of the oxygen electrode was routinely checked by measuring the uncoupled rate (i.e. the respiratory rate in the presence of the uncoupler (FCCP) at 0.2 µM) and the oxygen electrode was calibrated with the appropriate volume of oxygenated medium (i.e. medium equilibrated with air). The oxygen concentration of air-saturated medium at 37°C was assumed to be 406 nmol/ml (Reynafarje B *et al.* (1985). *Anal. Biochem.* 145, 406-418). Oxygen consumption was measured in the absence (state 4) and in the presence (state 3) of 250 µM ADP. The skeletal muscle mitochondria respiratory control ratio (state 3/state 4 oxygen consumption) was around 4.0 with succinate as substrate. For the measurements, 0.5 mg of mitochondrial protein per ml of assay medium (120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA and 0.3% defatted BSA, pH 7.2) was added to the oxygen electrode chamber followed by 5 µM rotenone, 1 µg/ml oligomycin and 4 mM succinate. Afterwards, adenosine, guanosine, cytidine, thymidine and uridine mono- di- and triphosphates were added to the oxygen electrode chamber at a concentration of 1 mM. The pH of each nucleotide solution was brought to 6-7 so that no modification in the pH of the reaction mixture occurred after their addition.

Measurement of proton leak.

The rate at which protons cycle across the mitochondrial inner membrane, which does not contribute to ATP synthesis by oxidative phosphorylation, is given by

the relationship observed between mitochondrial membrane potential and oxygen consumption rate during titration with electron transport chain inhibitors. This is a non-linear relationship, which suggests that the rate of dissipation of redox energy varies with membrane potential (Brown G.C. and Brand M.D. (1991) Biochim. Biophys. Acta 1059, 55-62). We determined the respiration rate and mitochondrial membrane potential simultaneously using oxygen electrodes and electrodes sensitive to the potential-dependent probe $TPMP^+$, and established the kinetic response of the proton leak to potential during titrations of the potential with inhibitors of electron transport (Brand M.D. (1995) Bioenergetics. A practical approach (Brown G.C. and Cooper C.E., eds.), IRL Press, pp. 39-62). For each run, 0.5 mg of mitochondrial protein per ml of assay medium (120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA and 0.3% defatted BSA, pH 7.2) was added to the oxygen electrode chamber. Prior to measurements the electrode was calibrated with sequential additions of up to 2 μM TPMP. 5 μM rotenone was added to prevent respiration on endogenous NAD-linked substrates. 1μg/ml oligomycin inhibited mitochondrial ATP synthase and 75 ng/ml nigericin was added to bring the difference in pH across the inner mitochondrial membrane close to zero. 4 mM succinate was used as substrate. Additions of malonate up to 2 mM were sequentially performed. At the end of each run, the uncoupler FCCP at 0.2 μM was added to dissipate the membrane potential, so that the TPMP was released by the mitochondria back to the medium. TPMP binding correction for skeletal muscle was taken to be 0.35 (μl/mg protein)$^{-1}$(Rolfe D.F.S. *et al.* (1994) Biochim. Biophys. Acta 1118, 405-416.).

Determination of free Mg^{2+} concentration.

Free Mg^{2+} concentration was determined from total Mg^{2+} in the assay medium. Apparent stability constants were calculated from absolute stability constants taken from (Fabiato A. and Fabiato A. (1979) J. Physiol. Paris, 75, 463-505) at a precise temperature and ionic strength.

RESULTS

The effects of adenosine, guanosine, cytidine, thymidine and uridine mono-di- and triphosphates at 1 mM on the state 4 respiration rate of rat skeletal muscle mitochondria were examined. In resting muscle, the physiological concentration of

ATP is 4 mM and of ADP is 0.013 mM. All nucleoside triphosphates as well as AMP showed an increase in the state 4 rate (41-75% for nucleoside triphosphates and 38% for AMP; figure 1). The nucleoside triphosphates all stimulated respiration by 60% in the absence of added Mg or EDTA, but this was entirely explained by chelation of endogenous contaminating Mg, which is a potent inhibitor of the basal proton conductance in muscle mitrochondria. Cadenas S., et al . 8th Int. Congr. Obesity, Paris. Abstract HTP10 (1998).

At Mg^{2+} concentrations in the assay medium up to 2 mM, no effect of any nucleotide on state 4 rate was found, except for 1 mM AMP, which again stimulated the rate. When EDTA, which chelates Mg^{2+} very efficiently (apparent binding constant equals 390788.33 M^{-1}, pH 7.2), was present at 1 mM in the assay medium, it abolished the effect of nucleoside triphosphates on respiration rate.

The observed increase in the respiration rate caused by the addition of 1 mM AMP was further confirmed by measuring the proton leak curves in the presence of 1 mM EDTA (figure 2). Figure 3 shows the respiration rate dependance on AMP concentration up to 2 mM (a) and up to 0.5 mM (b).

Both methods, state 4 respiration rate and proton leak measurements gave the same result: proton conductance in rat skeletal muscle mitochondria was stimulated by AMP, with half-maximal effects at about 100 μM.

FIGURE LEGENDS

Figure 1. Effect of nucleoside mono-, di- and triphosphates at 1 mM on rat skeletal muscle mitochondria respiration rate (% state 4). Data are mean ± SEM of three rats done in triplicate.

Figure 2. Proton leak curves in the presence or absence of 1 mM AMP (assay medium containing 1 mM EDTA). Data are mean ± SEM of three rats done in triplicate.

Figure 3. Effect of AMP from 0.25 to 2 mM (a) and from 0.05 to 0.5 mM (b) on rat skeletal muscle respiration rate (% state 4). Data are mean ± SEM of three rats done in triplicate.

Claims

1. A screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;

b) measuring the oxygen consumption; and

c) identifying compounds which increase oxygen consumption.

2. A method according to claim 1 which further comprises the steps of

a) contacting the compounds identified in claim 1 with mitochondria in the presence of a substrate for respiration in the presence of a buffer system and in the presence of AMP and measuring the oxygen consumption; and

b) comparing the oxygen consumption in claim 1 step (b) and claim 2 step (a) and identifying compounds where there is not an additive effect on oxygen consumption as compounds which activate the AMP-sensitive regulatory site.

3. A screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;

b) measuring the membrane potential; and

c) identifying compounds which decrease the membrane potential.

4. A method according to claim 3 which further comprises the steps of

a) contacting the compounds identified in claim 3 with mitochondria in the presence of a substrate for respiration in the presence of a buffer system and in the presence of AMP and measuring membrane potential, and

b) comparing the membrane potential in claim 3 step (b) and claim 4 step (a) and identifying compounds where there is not an additive effect on membrane potential as compounds which activate the AMP-sensitive regulatory site.

5. A screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;

b) measuring the oxygen consumption and measuring the membrane potential; and

c) identifying compounds which increase oxygen consumption and decrease the membrane potential.

6. A method according to claim 5 which further comprises the steps of

a) contacting the compounds identified in claim 6 with mitochondria in the presence of a substrate for respiration in the presence of a buffer system and in the presence of AMP, measuring the oxygen consumption and measuring the membrane potential; and

b) comparing the oxygen consumption and the membrane potential in claim 5 step (b) and claim 6 step (a) and identifying compounds where there is not an additive effect on oxygen consumption and membrane potential as compounds which activate the AMP-sensitive regulatory site.

7. A method according to any one of claims 1 to 6 wherein the mitochondria are isolated mitochondria.

8. A method according to any one of claims 1 to 6 wherein the mitochondria are skeletal muscle mitochondria.

9. A method according to claim 8 wherein the skeletal muscle mitochondria are rat skeletal muscle mitochondria.

10. A method according to any one of claims 1 to 6 wherein the mitochondria are present in intact eukaryotic cells.

11. A method according to claim 10 wherein the intact cells present in tissue slices of mammalian originor cell lines of mammalian origin.

12. A method according to any one of claims 1 to 6 wherein a complex 1 inhibitor is present.

13. A method according to any one of claims 1 to 6 wherein the substrate is a succinate salt.

14. A method according to claim 13 wherein rotenone is present.

15. A method according to any previous claim wherein the screening method is carried out in the presence of varying concentrations of an electron transport inhibitor.

16. A method according to claim 15 wherein the electron transport inhibitor is selected from a malonate salt, myxothiazol or cyanide salt.

17. A method according to claim 16 wherein the electron transport inhibitor is a malonate salt.

18. A screening method according to any one of claims 1, 2, or 5-17 wherein the oxygen consumption is measured by an oxygen electrode.

19. A screening method according to any one of claims 3 to 17 wherein the membrane potential is measured using ion selective electrodes.

20. A screening method according to any one of claims 3 to 17 wherein the membrane potential is measured using fluorescent membrane potential dyes.

21. A screening method according to any previous claim wherein an inhibitor of ATP synthesis is present.

22. A method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

a) contacting AMP or an analogue thereof with mitochondria in the presence of a substrate for respiration in the presence of a buffer system;

b) measuring the oxygen consumption and/or the membrane potential;

c) contacting AMP or an analogue thereof with mitochondria in the presence of a substrate for respiration in the presence of a buffer system in the presence of a known specific inhibitor of a mitochondrial protein;

d) measuring the oxygen consumption and/or the membrane potential;

e) identifying a protein whose inhibitor reduces the increase in oxygen consumption and/or the decrease in membrane potential caused by AMP as a protein which is involved in the AMP activated proton leak.

23. A method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

a) contacting AMP or an analogue thereof with mitochondria of modified protein composition in the presence of a substrate for respiration in the presence of a buffer system;

b) comparing the effect of AMP or a high affinity analogue on oxygen consumption and/or the membrane potential on mitochondria with modified protein composition with control mitochondria;

c) identifying modifications in protein composition which affect the increase in oxygen consumption and/or the decrease in membrane potential caused by AMP and hence identifying the corresponding protein/s which are involved in the AMP activated proton leak.

24. A method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

a) extracting the proteins from mitochondria and separating and purifying these proteins by methods known to those skilled in the art;

b) incubating the separated proteins with a fluorescent, radiolabelled or other labelled AMP or analogue in a suitable binding assay using methods known to those skilled in the art;

c) identifying those pure proteins which bind labelled AMP or analogue as being proteins involved in the AMP activated proton leak and

d) partially sequence the purified protein and using techniques known to those skilled in the art construct probes to allow the gene to be identified.

25. A method of identifying a protein which is involved in the AMP activated proton leak comprises the steps of:

a) extracting the proteins from mitochondria and incubating them with a photoaffinity labelled version of AMP or analogue using methods known to those skilled in the art to label proteins;

b) thereafter extracting, isolating and purifying those labelled proteins by methods known to those skilled in the art

c) identifying the structure of these proteins; and

d) identifying these proteins as proteins involved in the AMP activated proton leak; and

e) partially sequence the purified protein and using techniques known to those skilled in the art construct probes to allow the gene to be identified.

26. A screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

a) contacting a test compound with a protein which is involved in the AMP activated proton leak in a protein binding assay and identifying compounds with a high binding affinity as compounds which activate an AMP-sensitive regulatory site on mitochondria.

27. A method for identifying a gene which expresses a protein which is involved in the AMP activated proton leak comprising the steps of :

a) searching genomic databases and identifying genes which have an AMP binding domain;

b) expressing these genes in cells;

c) isolating the mitochondria from said cells; and

d) determining which mitochondria have an altered response to AMP (when compared to control) and hence determine which of these genes are responsible for the AMP activated proton leak.

28. A regulatory site for a mitochondrial proton leak wherein the site is activated by adenosine monophosphate (AMP).

29. The use of a regulatory site according to claim 28 in a screening assay to identify compounds which are useful in the treatment of obesity.

30. A regulatory site according to claim 28 which is a protein.

ABSTRACT

The present invention provides a screening method for the identification of compounds which activate an AMP-sensitive regulatory site on mitochondria comprising the steps of:

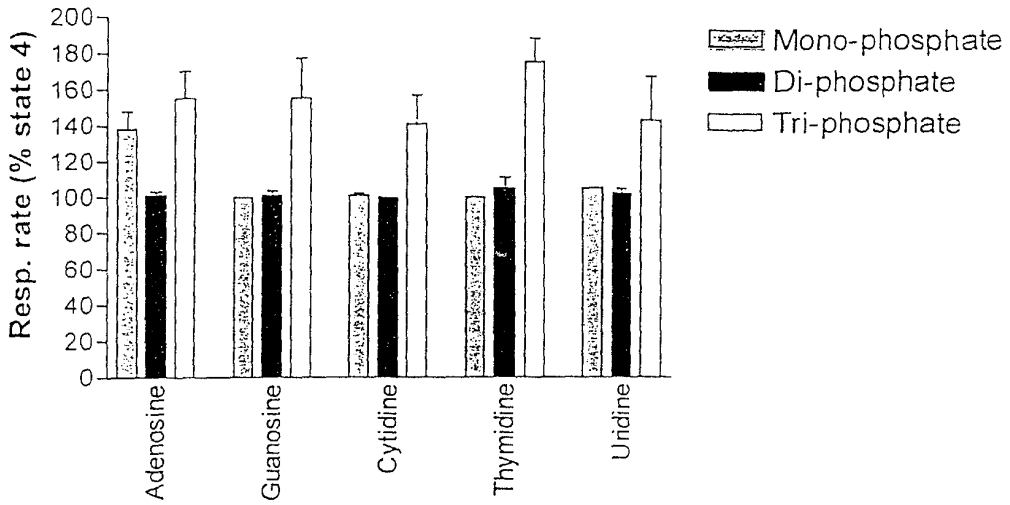
a) contacting a test compound with mitochondria in the presence of a substrate for respiration in the presence of a buffer system ;

b) measuring the oxygen consumption; and

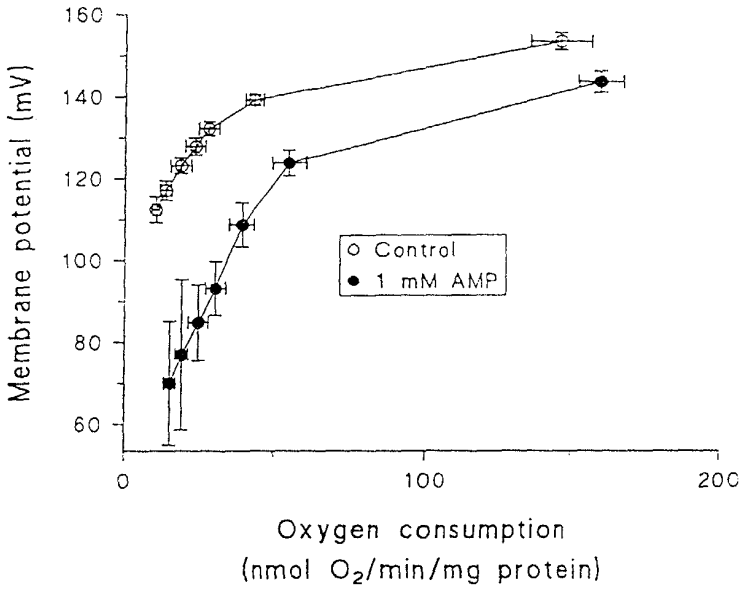
c) identifying compounds which increase oxygen consumption. Alternatively or additionally membrane potential can be measured.

The invention also comprises a regulatory site for a mitochondrial proton leak.

F. ..re 1. Effect of mono-, di- and triphosphate nucleosides at 1 mM on rat skeletal muscle mitochondria respiration rate (% state 4). Data are mean ± SD of three rats done in triplicate.

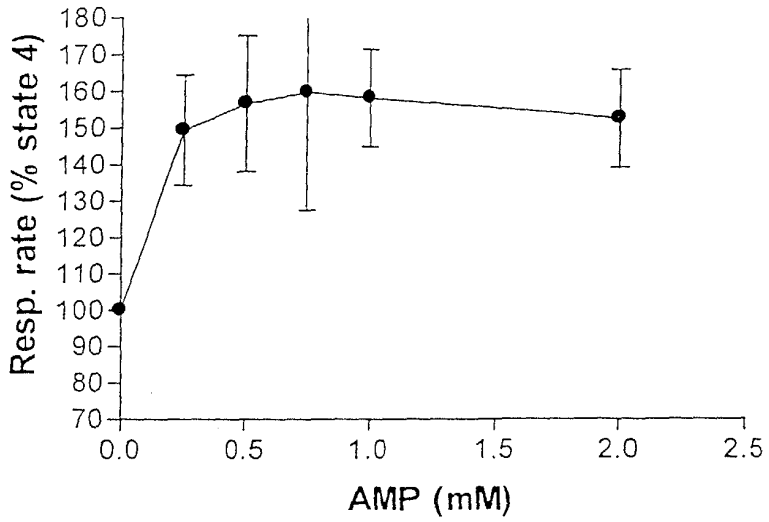


F re 2 Proton leak curves in the presence or absence of 1 mM AMP (assay medium containing 1 mM EDTA). Data are mean ± SD of three rats done in triplicate.



Fi. .e 3. Effect of AMP from 0.25 to 2 mM (a) and from 0.05 mM to 0.5 mM (b) on rat skeletal muscle respiration rate (% state 4). Data are mean ± SD of three rats done in triplicate.

a



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